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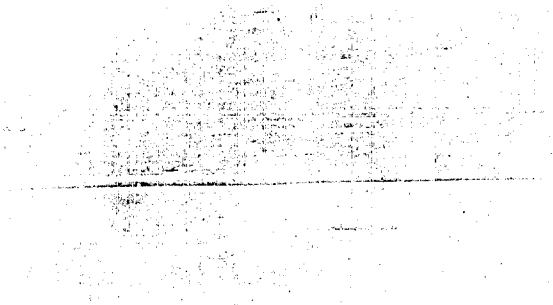

HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POISON

Second Quarterly Report of Progress

on

Research Project Number 4B04-14-004
Order Number FDO-5013

October 1 - December 31, 1960

Conducted by

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HAPTENIC PROPERTIES OF PARALYTIC SHELLFISH POSION

I. Introduction

The purpose of this project is (a) to determine the feasibility of joining the toxin of Gonyaulax catenella with other molecules to produce one or more conjugates having immunogenic properties, (b) to develop a specific micro-assay method for paralytic shellfish poison based on immunological reactions, and (c) to lay the ground work for immunization of humans against the poison. Although emphasis has been given almost exclusively to the first objective, the nature of the research has given rise to a number of observations suggesting ancillary studies related either to the chemistry of paralytic shellfish poison (PSP) or to the development of quantitative chemical assay procedures. Very little work has been undertaken along these lines because of the limited supply of the poison available.

In the first quarterly report evidence was presented to indicate that PSP reacts with nitrous acid and that this reaction product will couple with proteins. The failure of conjugated PSP-ovalbumin to elicit antibody responses in rabbits was attributed to the high toxicity of the preparation. It was evident at that time that the two immediate needs were (a) to develop techniques for decreasing the toxicity of the protein conjugates without seriously altering their immunogenic properties and (b) to develop quantitative techniques for following the reactions involved. It has been possible to partially fulfill the second need by following the color changes which take place in the coupling reaction.

This report summarizes the research conducted from October 1, 1960 to December 31, 1960. During this time the emphasis has been given to the conjugation of PSP with ovalbumin and bovine gamma II globulin and to the study of the immunological characteristics of the resulting compounds.

II. Experimental

Preparation of non-toxic PSP conjugated antigen.

Through a series of modification in the procedures described previously, PSP-conjugates of ovalbumin and bovine gamma II globulin have been produced, which were non-toxic when injected into mice at a level equivalent to at least 200 µg PSP. The preparations were well tolerated by rabbits when injected as antigens. The major difference between this method and that described previously is the inclusion of a dialysis step which appears to separate the uncoupled and toxic diazotized PSP from the conjugated protein.

Diazotized PSP was prepared by the action of excess sodium nitrite on PSP as follows: Three milliliters PSP solution (pH 2-4 containing 2.93 mg poison per ml) and 1/2 ml 0.036 mM NaBr were placed in a ten milliliter volumetric flask. A large excess of solid sodium nitrite (0.4 g) was dissolved in the solution, after which 0.5 ml 2M HCl was added and the flask stoppered. The reaction was allowed to proceed for 30 min. at 25°C. At the end of this period, the flask was placed in an ice bath and the contents diluted to 10 ml. The final concentration was equivalent to 0.879 mg PSP/ml.

The sodium bromide was added in an effort to suppress [illegible] (1), but subsequent studies have indicated that this addition [illegible] sary. It should also be recognized that the exact nature of the reaction with nitrite is not known and that the term "diazotized" is used at this time as a matter of convenience.

The conjugated antigens were prepared by combining 10 ml of diazotized PSP with 4 ml of 12% bovine gamma II globulin or ovalbumin at pH 7 and allowing the mixture to stand for 10 days at 25°C. The initial color of these preparations was pale yellow. In the case of the globulin reaction, a change from pale yellow to dark amber was observed with respect to time while the ovalbumin preparation changed from a pale to bright yellow. The controls for these reactions consisted of the protein solutions without diazotized poison and diazotized poison without proteins. At the end of the reaction period both the protein solutions were colorless and the diazotized poison remained a pale yellow.

The conjugated antigens and controls were placed in cellophane bags and dialyzed at 25°C against 8 hourly changes of distilled water with agitation. The first few dialysates from the conjugated proteins were faintly yellow, while those from the protein alone were colorless. The dialysates from the diazotized poison were discernibly yellow for the first six changes. A corresponding decrease in color of the poison solution was observed. After dialysis the conjugated PSP-bovine gamma II globulin was red-orange and PSP-albumin a deep yellow. Those solutions were diluted in physiological saline to contain 6.25 mg protein/ml and an estimated

concentration equivalent to 0.01 mg PSP/mg protein. Losses were estimated from the intensity of the yellow color of the diazotized poison recovered from the dialysate. In order to completely detoxify the PSP-albumin, it was necessary to dialyze this preparation for an additional six hours against running water.

The evidence developed at this time, indicating that PSP-protein conjugates have been formed, may be summarized as follows:

1. PSP-proteins have a characteristic color which is different from the color of the reactants.
2. The color associated with PSP-proteins is not dialyzable.
3. Precipitation of PSP-protein with trichloroacetic acid does not cause a dissociation of colored moiety.

An observation that PSP forms a colored reaction product in the presence of aromatic diazonium salts suggested the possibility of coupling PSP to protein through the use of a doubly diazotized intermediate, such as bis-benzidine. Since histamine has many similarities to PSP, including the capacity to react with aromatic diazonium salts, it is being used in the development of a model system.

The reaction between PSP and aromatic diazonium salts is very sensitive. Because large numbers of other compounds enter into similar reactions, it is an unattractive choice as a basis for an assay procedure when compared to the reaction between diazotized PSP and β-naphthol, which was discussed in the previous report.

Immunological Studies Related to Determining the Antigenicity of PSP-ovalbumin and PSP-bovine gamma II globulin.

The PSP-ovalbumin and PSP-bovine gamma II globulin, described in the previous section, were used to immunize four groups of three rabbits each. The first group received intravenous injections (lateral ear vein) of PSP-bovine gamma II globulin (PSP-globulin), and the second group received intravenous injections of PSP-ovalbumin. The immunization schedule employed is displayed in Table 1.

The remaining two groups of rabbits were immunized by intramuscular injection (semimembranosus and semitendenosus muscles) with a mixture of equal proportions of Freund's adjuvant and the coupled antigens. Three and one-tenth ml. of these mixtures were injected in each hind leg. These rabbits were bled 21 days after injection. The total volume of the PSP-protein solutions used to immunize the rabbits in each of the four above groups was 3.1 ml.

Four additional groups of three rabbits each were immunized intravenously or intramuscularly, as described above, with ovalbumin and bovine gamma II globulin. These rabbits served as a source of anti-gamma globulin and anti-ovalbumin sera for control purposes and as means of noting which of the two immunization routes elicited the highest antibody titers.

The collected sera from the above rabbits were tested for their precipitin content by mixing a constant volume of serum with varying concentrations of antigen, incubating in a 37°C water bath for 1 hour, followed

by overnight incubation at 5°C. Following incubation the tubes were centrifuged at 1000 rpm for 3 minutes and observed for precipitate. In all tests, controls consisted of 0.4 ml. of saline mixed with 0.5 ml. of serum diluted 1:5, and 0.4 ml. of saline mixed with 0.4 ml. of each of the undiluted antigens. In none of the titrations did a precipitate form in the control tubes. The antibody titers of the anti-globulin, anti-PSP-globulin, anti-ovalbumin and anti-PSP-ovalbumin sera were all in excess of 1:2,560 when tested against their homologous antigens, indicating excellent antibody production.

Tables 2 through 6 show the results observed when anti-PSP-globulin and anti-globulin sera were titrated against PSP-globulin, globulin, and PSP-ovalbumin antigens. Sera 9, 12, 13, and 14 were obtained from rabbits injected intramuscularly and sera 15, 18, 19, and 20 were from rabbits injected intravenously. Though six rabbits were injected with globulin (3 I.V. and 3 I.M.), only two of these sera were titrated in order to conserve the limited supply of conjugated antigens. (See Tables 4 and 6, sera 9 and 15.)

To determine whether antibody was produced to the PSP portion of the molecule, cross reactions were attempted with the heterologous systems of anti-PSP-globulin to PSP-ovalbumin and anti-PSP-ovalbumin serum to PSP-globulin. The results obtained with serum number 12 (Table 2) indicated that some cross reaction occurred between PSP-ovalbumin and anti-PSP-globulin. However, when these tests were repeated, the cross reaction was not observed (Table 3).

Tables 7 through 10 show the results observed when anti-PSP-ovalbumin and anti-ovalbumin sera were titrated against PSP-ovalbumin, ovalbumin and PSP-globulin. Serum number 29 (anti-PSP-ovalbumin) consistently cross reacted with PSP-globulin in a number of trials. (See Tables 9 and 10.) Since the only factor held in common between the two systems was the PSP portion of the molecule, and since normal serum, collected from this rabbit prior to immunization with PSP-ovalbumin, did not react with any of the antigens, a haptenic response appears to have been obtained in this instance. In this series, also, six rabbits were immunized with ovalbumin, but only two sera were titrated in order to conserve conjugated antigens (Sera 24 and 30, Tables 8 and 11).

In addition to tube precipitin tests, a series of Ouchterlony plates were prepared in which anti-PSP-globulin, anti-PSP-ovalbumin, anti-globulin, and anti-ovalbumin were tested against homologous and heterologous antigens. A second series of plates was prepared in which 5 mouse lethal units of PSP were reacted against the above four types of sera as well as normal serum obtained from the same rabbits prior to immunization. The results of these tests are summarized in Tables 12 and 13. Except for the observations on serum #29, these data are in agreement with the results obtained from the tube precipitin tests and indicate that strong antibody responses were produced only to the protein portions of the conjugate molecules.

Because it is possible to obtain non-precipitating types of antibodies which are not demonstrable by <u>in vitro</u> tests, a series of mouse protection tests were conducted to determine the possibility of protective

antibodies having been produced. Three pairs of mice were employed and injected intraperitoneally with 1.0 ml. of the following: saline, normal rabbit serum, and anti-PSP-ovalbumin (serum 29). Twenty-four hours later the mice received a second 1.0-ml. injection of their respective reagents and were challenged 4 hours later with 5 lethal mouse units of PSP. All the mice died between 3 1/5 and 4 1/2 minutes. Because no significant difference in time of death was observed between the mice receiving saline or normal serum as compared to those receiving immune serum, it was assumed that protective antibodies were not produced.

As an alternate procedure for detecting antibodies not demonstrable by in vitro techniques, a skin test was performed to demonstrate the presence of sensitizing antibodies. Rabbit No. 27 (immunized with PSP-ovalbumin) was depilated on the dorsal surface and injected intradermally at 5 sites with 0.1 ml. aliquots of the following materials:

(a) ovalbumin

(b) PSP-ovalbumin

(c) globulin

(d) PSP-globulin

(e) 1 mouse unit PSP

Observations were made at various intervals throughout a 48-hour period and a very strong Arthus-type reaction was observed at the sites in which ovalbumin and PSP-ovalbumin were injected. No reactions were observed for the remaining materials.

Because of the consistent precipitin reaction between anti- [illegible] ovalbumin and PSP-globulin (Tables 9 and 10), it appears that PSP may be haptenic, but that insufficient toxin was coupled to the protein to obtain a specific reaction of high titer. These data suggest that a more complete saturation of the protein molecules with toxin may result in the production of sera with which the haptenic properties of the PSP can be demonstrated more clearly.

III. Conferences

A. BERKELEY, CALIFORNIA

An informal meeting was held December 5, 1960, on the Berkeley campus, University of California, to discuss chemical and immunological reactions of paralytic shellfish poison. Those in attendance were:

Dr. Henry Rapoport, Department of Pharmaceutical Chemistry
University of California

Dr. John H. Phillips, Department of Bacteriology
University of California

Dr. K. H. Lewis, Chief, Milk and Food Research, Taft Engineering Center, Public Health Service

Dr. J. E. Campbell, Chief, Food Chemistry, Milk and Food Research, Taft Engineering Center, Public Health Service

Mr. Joseph F. O'Brien, Senior Sanitarian (Shellfish Consultant), Region IX, Public Health Service, San Francisco, California

During the course of this discussion several useful ideas were developed which have been summarized below.

1. Utilization of the basic characteristics of PSP for coupling reactions.

Formation of stable PSP-protein conjugates might be induced by reacting the base form of PSP with an acid protein. Although the nucleoproteins would be a natural choice for this reaction, other proteins could be made acid by the addition of formaldehyde prior to coupling.

2. Modification of the reaction between diazotized PSP and proteins.

The lengthy time required for coupling diazotized PSP and proteins was considered to be very undesirable. It appeared that the time required for this reaction would be greatly reduced by raising the pH of the reactant to 9 - 9.5. It was also suggested that coupling should be carried out under an inert atmosphere in order to minimize oxidative changes.

3. Alternate methods for immunization.

In discussing the relative merits of various immunization techniques the possibility of inducing an immune reaction against PSP by the injection of the organism Gonyaulaux catenella was suggested. It was felt that direct injections of homogenates of the cells would be tolerated by rabbits. If it proved to be too toxic, the homogenates could be fractionated by dialysis or ammonium sulfate fractionation.

B. CINCINNATI, OHIO

On December 31, 1960, an informal meeting was held between Dr. Dudley P. Glick and the members of the Milk and Food Research Staff involved in the project. Informal progress reports were given by Drs. Campbell and Angelotti on the chemical and immunological phases of the work. These reports were

followed by a general discussion including an attempt to evaluate our current position in terms of the major objectives.

It was generally recognized that the limited availability of purified PSP represents a substantial handicap, especially in connection with undertaking an investigation needed for the development of a clear understanding of the chemical reaction involved or for exploring promising observations which might lead to a useful micro-chemical assay. Essentially all the work reported to date has been accomplished using 129 milligrams of purified PSP and about half of this was used for the preparation of the protein conjugates. Sufficient PSP is available to make one more attempt at immunization and to evaluate the haptenic property of the diazotized PSP. Further progress will depend on obtaining additional supplies of purified PSP from the U. S. Army Chemical Corps Biological Laboratory.

IV. Projected Research for Third Quarter, FY 1961

Projected research for the third quarter, FY 1961, will be directed toward the following objectives:

1. Preparation of PSP-ovalbumin and PSP-bovine gamma II globulin in which the amount of diazotized PSP coupled to the protein is increased by a factor of 10. (The suggestions of Dr. Rapoport in regard to increasing the rate of reactions will be included in these syntheses.)
2. Investigation of the immunological properties of the above preparations to determine whether or not the PSP portion of the molecule is a haptene.
3. Development of more rigorous evidence concerning the nature of the PSP-

proteins through the use of physical techniques such as electrophoresis and ultracentrifugation.

4. Continued studies on the development of chemical micro-assay procedures for PSP.

5. Investigation of alternate methods for coupling proteins to PSP.

V. Summary

Through modifications of procedures described previously, a technique has been developed for conjugating diazotized PSP to protein so that the final product is of sufficiently low toxicity to be suitable for use as an antigen.

Immunological investigations of PSP-ovalbumin and PSP-bovine gamma II globulin reveal that both onjugates will elicit an antibody response in rabbits. Although it was not possible to demonstrate clearly that the PSP portion of the molecule had haptenic properties, evidence was developed suggesting this to be the case.

VI. References

1. Brown, R.D., Duffin, H.C., Maynard, J.C., Ridd, J.H. The Mechanism of the Coupling of Diazonium Salts with Heterocyclic Compounds. Part I. Glyoxaline. J. Chem. Soc. 3937. 1953.

Table 1.

Immunization schedule for rabbits receiving intravenous injections of globulin, ovalbumin, PSP-globulin and PSP-ovalbumin

Day	Dose
1	0.1 ml.
3	0.2 ml.
5	0.3 ml.
8	0.5 ml.
10	1.0 ml.
17	Bled
19	1.0 ml.
26	Bled

Table 2.

Reaction of rabbit sera, produced by intramuscular injection of PSP-globulin mixed in equal proportions with Freund's adjuvent, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Un-dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Globulin IM first bleeding	PSP-Globulin	12	+	+	+	2+	+	2+	2+	2+	+	±
		13	+	+	2+	+	2+	3+	4+	4+	4+	3+
		14	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+
	Globulin	12	-	+	+	2+	2+	2+	3+	3+	2+	+
		13	-	-	-	+	+	2+	3+	4+	4+	4+
		14	2+	+	2+	4+	4+	4+	4+	4+	4+	4+
	PSP-Ovalbumin	12	3+	+	±	-	-	-	-	-	-	-
		13	+	-	-	-	-	-	-	-	-	-
		14	+	-	-	-	-	-	-	-	-	-

Table 3.

Reaction of rabbit serum number 12, produced by intramuscular injection of PSP-globulin mixed in equal proportions with Freund's adjuvent, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Globulin IM first bleeding (repeat of 12)	PSP-Globulin	12	+	+	+	+	+	2+	3+	3+	2+	2+
	Globulin	12	-	-	-	+	+	2+	3+	4+	4+	3+
	PSP-Ovalbumin	12	-	-	-	-	-	-	-	-	-	-

Table 4.

Reaction of rabbit serum, produced by intramuscular injection of globulin mixed in equal proportions with Freund's adjuvent, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Globulin IM	PSP-Globulin	9	4+	4+	4+	4+	4+	4+	4+	3+	2+	-
	Globulin	9	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+
	PSP-Ovalbumin	9	-	-	-	-	-	-	-	-	-	-

Table 5.

Reaction of rabbit sera, produced by intravenous injections of PSP-globulin, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Un. dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Globulin IV first bleeding	PSP-Globulin	18	+	+	+	+	2+	2+	2+	+	+	-
		19	±	±	+	+	+	+	+	3+	+	+
		20	Rabbit died - unknown causes									
	Globulin	18	-	-	+	2+	3+	3+	3+	4+	2+	2+
		19	-	-	-	+	+	+	2+	2+	2+	2+
		20	Rabbit died - unknown causes									
	PSP-Ovalbumin	18	+	-	-	-	-		-	-	-	-
		19	+	-	-	-	-	-	-	-	-	-
		20	Rabbit died - unknown causes									
Anti-PSP-Globulin IV second bleeding	PSP-Globulin	18	4+	4+	4+	4+	4+	4+	4+	3+	3+	+
		19	4+	4+	4+	4+	4+	4+	4+	4+	3+	+
		20	Rabbit died - unknown causes									
	Globulin	18	-	+	2+	2+	3+	4+	4+	3+	3+	3+
		19	+	4+	4+	4+	4+	4+	4+	4+	4+	4+
		20	Rabbit died - unknown causes									
	PSP-Ovalbumin	18	-	-	-	-	-	-	-	-	-	-
		19	+	-	-	-	-	-	-	-	-	-
		20	Rabbit died - unknown causes									

Table 6.

Reaction of rabbit serum, produced by intravenous injections of globulin, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-Globulin IV first bleeding	PSP-Globulin	15	4+	4+	3+	3+	2+	2+	+	+	-	-
	Globulin	15	4+	4+	4+	4+	3+	3+	3+	2+	2+	+
	PSP-Ovalbumin	15	2+	-	-	-	-	-	-	-	-	-
Anti-Globulin IV second bleeding	PSP-Globulin	15	4+	3+	3+	3+	3+	3+	2	2+	2+	+
	Globulin	15	+	4+	4+	4+	4+	4+	4+	4+	4+	4+
	PSP-Ovalbumin	15	-	-	-	-	-	-	-	-	-	-

Table 7.

Reaction of rabbit sera, produced by intramuscular injection of PSP-ovalbumin mixed in equal proportions with Freund's adjuvent, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Un-dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Ovalbumin IM	PSP-Ovalbumin	21		-	±	+	2+	4+	4+	4+	4+	4+
		22		-	-	-	+	3+	4+	4+	4+	3+
		23		+	2+	4+	4+	4+	4+	4+	4+	4+
	Ovalbumin	21		-	+	4+	4+	4+	4+	4+	4+	3+
		22		-	-	+	4+	4+	4+	3+	2+	+
		23		+	4+	4+	4+	4+	4+	4+	3+	2+
	PSP-Globulin	21		-	-	-	-	-	-	-	-	-
		22		-	-	-	-	-	-	-	-	-
		23		-	-	-	-	-	-	-	-	-

Table 8.

Reaction of rabbit serum, produced by intramuscular injection of ovalbumin mixed with equal proportions of Freund's adjuvent, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Un-dil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-Ovalbumin IM	Ovalbumin	24		-	+	2+	4+	4+	4+	4+	3+	+
	PSP-Ovalbumin	24		-	-	-	-	+	2+	3+	4+	3+
	PSP-Globulin	24		-	-	-	-	-	-	-	-	-

Table 9.

Reactions of rabbit sera, produced by intravenous injections of PSP-ovalbumin, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Ovalbumin IV first bleeding	PSP-Ovalbumin	27	-	-	+	2+	2+	3+	4+	4+	4+	4+
		28	-	+	+	+	2+	2+	3+	4+	4+	3+
		29	-	-	-	-	-	2+	4+	4+	4+	3+
	Ovalbumin	27	+	-	-	+	2+	2+	3+	3+	3+	2+
		28	+	+	+	+	2+	3+	3+	4+	3+	2+
		29	+	-	-	+	2+	3+	4+	4+	4+	2+
	PSP-Globulin	27	+	-	-	-	-	-	-	-	-	-
		28	-	-	-	-	-	-	-	-	-	±
		29	+	+	+	-	-	-	-	-	-	-
Anti-PSP-Ovalbumin IV second bleeding	PSP-Ovalbumin	27		-	-	-	-	±	+	2+	3+	3+
		28		-	-	-	-	±	+	2+	3+	2+
		29	Rabbit died - unknown causes									
	Ovalbumin	27		-	-	-	-	+	2+	3+	2+	+
		28		-	-	-	+	2+	2+	3+	2+	+
		29	Rabbit died - unknown causes									
	PSP-Globulin	27		-	-	-	-	-	-	-	-	-
		28		-	-	-	-	-	-	-	-	-
		29	Rabbit died - unknown causes									

Table 10.

Reaction of rabbit serum number 29 produced by intravenous injections of PSP-ovalbumin to PSP-globulin

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-PSP-Ovalbumin first bleeding IV 29	PSP-Globulin	29	+	+	+	±	-	-	-	-	-	-

Table 11.

Reaction of rabbit serum, produced by intravenous injections of ovalbumin, to various antigens

Serum diluted 1:5 0.4 ml./tube	Antigen	No. of Serum	Dilutions of Antigen 0.4 ml./tube									
			Undil.	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Anti-Ovalbumin IV first bleeding	Ovalbumin	30	4+	-	2+	3+	4+	4+	4+	4+	4+	4+
	PSP-Ovalbumin	30	2+	-	-	-	-	-	±	+	4+	4+
	PSP-Globulin	30		-	-	-	-	-	-	-	-	-
Anti-Ovalbumin IV second bleeding	Ovalbumin	30		+	4+	4+	4+	4+	4+	4+	3+	2+
	PSP-Ovalbumin	30		-	-	-	+	2+	4+	4+	4+	3+
	PSP-Globulin	30		-	-	-	-	-	-	-	-	-

Table 12.

Reaction of rabbit sera to various antigens as demonstrated in the Ouchterlony plate method

Content of center well 0.5 ml.	Days incubation at 5°C	Contents of peripheral wells			
		(A) Ovalbumin 0.5 ml.	(B) Globulin 0.5 ml.	(C) PSP-Globulin 0.5 ml.	(D) PSP-Ovalbumin 0.5 ml.
Anti-PSP-Globulin #12	2	No reaction	Heavy PPT	Heavy PPT	No reaction
	4	"	"	"	"
	6	"	"	"	"
	8	"	"	"	"
	10	"	"	"	"
Anti-PSP-Ovalbumin #29	2	Slight PPT	No reaction	No reaction	Slight PPT
	4	"	"	"	"
	6	Mod. PPT	"	"	Strong PPT
	8	"	"	"	"
	10	Strong PPT	"	"	"
Anti-Globulin #15	2	No reaction	No reaction	No reaction	No reaction
	4	"	Slight PPT	"	"
	6	"	Strong PPT	"	"
	8	"	Double Ring	"	"
	10	"	"	Heavy PPT	"
Anti-Ovalbumin #30	2	No reaction	No reaction	No reaction	No reaction
	4	Heavy PPT	"	"	"
	6	"	"	"	"
	8	"	"	"	Heavy PPT
	10	"	"	"	"

Table 13.

Reaction of rabbit sera to paralytic shellfish toxin as demonstrated in the Ouchterlony plate method

Content of center well	Days incubation at 5°C	Contents of peripheral wells			
		(A) 0.5 ml. Anti-Ovalbumin #30	(B) 0.5 ml. Anti-Globulin #15	(C) 0.5 ml. Anti-PSP-Ovalbumin #29	(D) 0.5 ml. Anti-PSP-Globulin #12
5 mouse units of PSP in 0.5 ml.	2	No reaction	No reaction	No reaction	No reaction
	4	"	"	"	"
	6	"	"	"	"
	8		"	"	"
	10	"	"	"	"
		Normal Serum #30	Normal Serum #15	Normal Serum #29	Normal Serum #12
	2	No reaction	No reaction	No reaction	No reaction
	4	"	"	"	"
	6	"	"	"	"
	8	"	"	"	"
	10	"	"	"	"